

SELECTIVE METABOLIC LABELING OF AXONAL PROTEINS; AN IN VITRO APPROACH FOR THE SEARCH OF PROTEINS WITH A ROLE IN DEVELOPMENT OR REGENERATION OF AXONS

HABILITATIONSSCHRIFT

zur Erlangung der Venia legendi
an der Medizinischen und Philosophischen Fakultät II
der Universität Zürich

vorgelegt von

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Winterthurerstrasse 190

CH-8057 Zürich / Switzerland

Zürich 1987

Zentralstelle der Studentenschaft



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CONTENTS

1. Introduction
2. A compartmental cell culture system for controlled variation of the axon's local environment: Rationale and experimental outline.
3. Neurons in the compartmental cell culture system express distinctive morphological features and respond in a highly differential manner to environmental stimuli.
 - 3.1. Axons of distinct neuronal subpopulations have distinct morphology.
 - 3.2. Neuronal electrical features and synaptic transmission are expressed in the compartmental cell culture system.
 - 3.3. The pattern of fasciculation of DRG axons is specifically modulated by co-cultured glia.
4. The compartmental cell culture system as a tool for the selective study of the expression of axonal proteins.
 - 4.1. Selective analysis of the expression of axonal proteins.
 - 4.2. The study of local axon-glia and axon-neuron interaction.
 - 4.3. Continued conditioning in studies of humoral glia-derived factors on axonal behavior and protein expression.
 - 4.4. Unequivocal identification of proteins secreted from axons of distinct neuronal subpopulations.

5. Examples of application in the search for proteins with a role in axon development of regeneration

5.1 Protein differences between axonal subpopulations may point to proteins involved in subpopulation-specific functions.

5.2 The expression of some axonal proteins is differentially modulated by non-neuronal cells of the peripheral and central nervous system.

5.3 Experimentally evoked modulation of axonal proteins as a tool for the identification of proteins that could be involved in concomitantly modulated axonal functions.

6. Concluding remarks.

7. Summary.

Acknowledgements

Legends.

1. INTRODUCTION

The complexity of the highly specific intercellular network that characterizes the nervous system is based mainly on two organizational principles, the multiplicity of the connections carrying informations between neurons or groups of neurons, on the one hand, and the functional diversification of neuronal cells, on the other. The morphological complexity of the neuronal network is implemented by the branching pattern of neuritic and dendritic extensions. The functional diversification is based upon differences in the ion channel composition of the neuronal plasma membranes and in the type of neurotransmitter secreted at the axons terminals.

The development of the highly complex network of interacting neurons depends on the capability of axons to extend their axons over long distances along specified pathways to make specific contacts with target neurons. The axonal functions that implement this network formation are phenomenologically described as elongation, sprouting, pathfinding, fasciculation, synthesis of a specific neurotransmitter and synapse formation. Some axonal functions, such as fasciculation (Bray et al., 1980; Raper et al. 1983a), pathfinding (Landmesser, 1980, 1984; Raper et al., 1983b) and synapse formation (Fuchs et al., 1981) involve features specific to neuronal subpopulations. Furthermore, evidence for differences among neurons of the same subpopulation, viz. motoneurons of different segmental origin, has come from studies on the development of specific motor pathways (for a review cf. Landmesser, 1980, 1984). A number of axonal functions are epigenetically regulated by the axons' cellular and humoral environment (Ramon y Cajal, 1928; Levi-Montalcini, 1954; Sperry, 1963; Patterson, 1978; Letourneau, 1982). Hence, features specific to neuronal

subpopulations and the neurons' capability to shape the behavior of their axons in response to environmental influences do seem to contribute significantly to the generation of the organizational order of the nervous system. However, relatively little is known regarding the molecular entities underlying axonal behavior. The overwhelming complexity of the nervous system has made the molecular study of the principles of its organization a difficult and tedious task. In the absence of a generally applicable approach to the determination of the macromolecular composition of neurons or axons, our knowledge on the distinct macromolecular differences between neuronal subpopulations was thus far derived mainly from cell surface binding studies of lectins and antibodies and the detection of transmitter-related and other enzymes (for more detailed discussion cf. chapter 5). Many approaches to the understanding of the principles underlying brain function have been undertaken on experimental systems of reduced complexity, one of these being neural tissue culture. Indeed, the cultivation of a large number of neuronal subpopulations from different locations and with different properties has been reported in the literature (for a review cf. Fischbach & Nelson, 1977). However, in most of the neuronal cell culture systems, the non-neuronal cells have not been eliminated and for many of them, they may be essential for optimal survival of the neurons. Hence, dissociated neuronal cell cultures, in most cases, cannot be used to establish a protein inventory of a particular neuronal subpopulation because of the inevitable presence of non-neuronal cells. However, the unique morphology of axons as long processes extending from the neuronal cell soma, together with the fact that the axonal proteins are synthesized in the cell soma and subsequently, by axonal transport, moved down the axon to their site of destination, can be exploited for an experimental approach to the selective analysis of axonal

proteins even when the axons are embedded between non-neuronal cells. This approach is based on a recently developed compartmental cell culture system that allows separate access to neuronal somas and to their axons (Campenot, 1977). When neurons are cultured in the center chamber of this compartmental cell culture system, their axons cross beneath a barrier into the side compartments whereas the cell somas are retained in the center compartment. This experimental set-up allows selective metabolic labeling of axonally transported proteins by adding a radioactive amino acid to the culture compartment that contains the neuronal somas and harvesting axons from the adjacent compartment (Sonderegger et al., 1983, 1984). Axonal proteins of different neuronal subpopulations or of axons exposed to a different cellular or humoral environment may then be separated by two-dimensional gel electrophoresis and individually quantified by computerized gel image analysis (Sonderegger et al., 1983, 1984, 1985). Applying this approach to two neuronal subpopulations, such as dorsal root ganglia or ventral spinal cord neurons, more than 1000 proteins were found in axons (P. Sonderegger, P.F. Lemkin, L.E. Lipkin and P.G. Nelson, submitted for publication).

Since the function of the vast majority of the axonal proteins is unknown, it seems obvious that, at the present time, the identification of the proteins involved in a particular axonal function cannot be approached in a random way. On the working assumption that the proteins transported into axons from cell bodies are selectively those employed in axonal functions and that the expression of axonal functions is based on the specific content of macromolecular building blocks, we screened the bulk of probably thousands of axonal proteins for such that attracted attention by being specific to neuronal subpopulations or whose

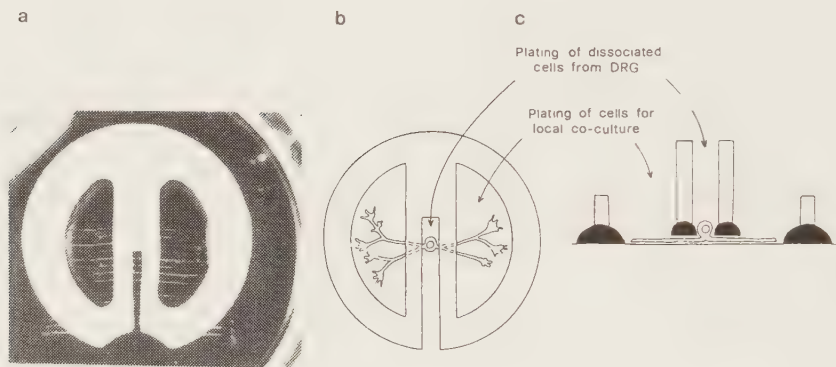
expression was subject to environmental modulation. Such proteins may be considered candidates for a role in particular axonal functions. In providing a hook for grappling with other proteins involved in the implementation of the same or related axonal functions or their regulatory apparatus, this approach provides a tool to the assignment of functions to axonal proteins.

In this paper, this generally applicable approach to the dissection of the cellular and molecular complexity of the nervous system is outlined. As examples of its application, the identification of axonal proteins that are specific for neuronal subpopulation and the identification of proteins that are under environmental control are reviewed with some detail.

2. A COMPARTMENTAL CELL CULTURE SYSTEM FOR CONTROLLED VARIATION OF THE AXONS' LOCAL ENVIRONMENT: RATIONALE AND EXPERIMENTAL OUTLINE

Cell cultures have been introduced for the study of neurobiological questions because they allow close morphological and functional investigation of the cells and a high degree of control over environmental conditions (Fischbach & Nelson, 1977). However, in dissociated cell cultures, the cells are exposed to the same environment in their entirety, a condition that for many cells (and particularly for neurons) does not correspond to the in vivo situation. As an example, axons of the neuronal cell somas in the dorsal root ganglia (DRG), are initially entirely surrounded by a peripheral glial environment. Upon entrance of the spinal cord, they come into contact with central glia. Furthermore, once in the spinal cord, the growing axons eventually meet a variety of spinal cord neurons. Hence, the changing environment of the outgrowing axons during their growth is in marked contrast to the positional constancy of the neuronal somas in the ganglia. Environmental regulatory influences are expected to be conveyed to the axons locally. However, the constant environment in the ganglion, with its own regulatory influences on the neuronal cell soma and the initial part of the axon (Mudge, 1984), may be essential for adequate differentiation of the axon as well. To imitate the in vivo situation with respect to the cellular environment of axons and neuronal somas while still exploiting the advantages of the in vitro approach, neurons were grown in a compartmental cell culture allowing independent control of the local environment of neuronal somas and neurites. Among various approaches to this goal, the system devised by Campenot (1977, 1979) has proven most practicable in our hands. In this system, three chambers are

formed by a Teflon divider (Fig. 1) set in a cell culture dish so that the compartments are connected by a thin film of medium with no bulk flow of liquid (for experimental details cf. Fig. 1). By virtue of the relatively slow dif-



Legend to Fig. 1

The compartmental cell culture system as originally devised by Campanot. (1977 & 1979). To give the outgrowing axons direction, about 15 parallel scratches, about 0.5 mm apart, were made across the surface of a collagenized, dry 35-mm cell culture dish. A drop of a film forming medium containing hydroxypropyl methylcellulose was deposited in the middle of the scratches and the Teflon inset, covered on its bottom side with silicon high vacuum grease, was placed into the dish so that the scratches spanned all three compartments. The system was tested for absence of hydrostatic bulk flow between the compartments by filling the two side compartments with 0.5 ml of growth medium whereas the center compartment was left empty. Only those plates that had no leakage of medium into the center compartment during 4 to 6 hours were used.

a) Top view of the Teflon inset.

b,c) Schematic drawing of the compartmental cell culture system, top view, and cross-sectional view, respectively.

fusion of medium through the liquid film connecting the compartments (cf. Sonderegger et al., 1983 & 1984), medium conditions can be maintained differently in each compartment. When neurons are plated in the center compartment, the axons grow out and are able to extend along the thin film of medium connecting the compartments into the side compartments, whereas the cell somas are retained in the center compartment. These features have been exploited previously for the study of the local effects of nerve growth factor (Camenot, 1977) and of the retrograde axonal transport of a variety of molecules including the neurotransmitter noradrenalin (Schwab and Thoenen, 1983) and tetanus toxin (H. Bigalke, personal communication). In the studies summarized here this system was used to obtain insight into the specific molecular composition of axons belonging to different neuronal subpopulations as well as to study the effects of the axons' local cellular microenvironment on the expression of axonal proteins.



3. NEURONS IN THE COMPARTMENTAL CELL CULTURE SYSTEM EXPRESS DISTINCTIVE MORPHOLOGICAL FEATURES AND RESPOND IN A HIGHLY DIFFERENTIATED MANNER TO ENVIRONMENTAL STIMULI

One of the principal questions raised in the context of a cell culture approach is whether cell cultures are adequate models for in vivo systems. A host of evidence has been reported on neuronal cell cultures, indicating that many cellular features of neurons are maintained in culture (for a review see Fischbach & Nelson, 1977). However, in exchange for the reduced complexity and increased accessibility of dissociated neuronal cultures, expression of features based on the organotypic complexity of the nervous system cannot be expected.

In our laboratory we have grown two distinct populations of neurons in the compartmental cell culture system: DRG neurons and neurons from the ventral half of spinal cord (VSC). The DRG neurons were exposed to local co-culture with a variety of cells, such as non-neuronal cells from the peripheral nervous system, non-neuronal cells from the central nervous system, and cells from the ventral spinal cord. A series of observations made during these studies indicate that neurons maintain a high degree of differentiation and cell type specific behavior when they are grown in culture.

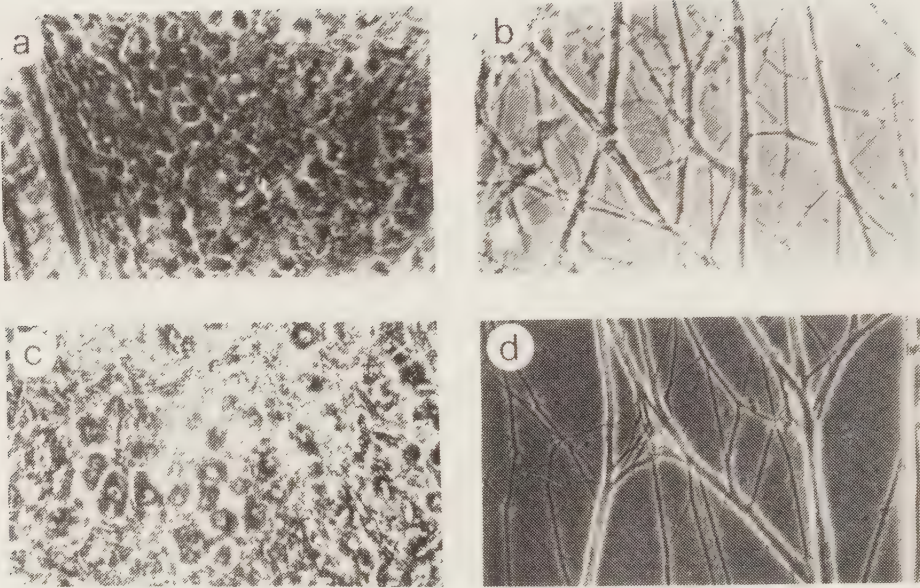
3.1. Axons of distinct neuronal subpopulations have distinct morphology.

Axons from different neuronal subpopulations have a variety of structural and functional features in common. There are, however, a number of morphological and functional characteristics that clearly distinguish axons of different neuro-

nal populations from each other. For example, a substantial proportion of cultured spinal cord neurons form cholinergic synapses with cultured myotubes (Fischbach, 1972), whereas DRG neurons make noncholinergic synaptic connections with central neurons (Ransom et al., 1977). During development, spinal cord and dorsal root ganglia neurons show distinct behavior with respect to pathfinding, fasciculation and selection of target cells. When grown in the compartmental cell culture system, neuronal cell somas and axons of DRG and VSC neurons were clearly distinct (Fig. 2a,b and c,d, respectively). DRG neurons, for the most part, had large cell somas and, on collagen-coated cell culture dishes, extensive fasciculation of their axons was observed. In contrast, the majority of VSC neurons were distinctly smaller their axons had a greater tendency to branch out of the fascicles.

3.2. Neuronal electrical features and synaptic transmission are expressed in the compartmental cell culture system.

Electrical stimulation of the DRG axons that crossed beneath the barrier separating the center and side compartments results in antidromic activation of action potentials in the DRG somas (Fig. 3a). To determine whether the axons of the side compartment were able to establish synaptic connections, spinal cord neurons, their natural target neurons, were co-cultured. Spinal cord cells, containing neurons and central non-neuronal cells were obtained from the ventral half of 6 day old chick embryos as described previously (Sonderregger et al., 1984). After tryptic dissociation, approximately 15,000 cells were directly plated onto axons in the side compartments. Four days after plating, the VSC cells were large enough to be impaled with high resistance glass microelectrodes. Continuous sponta-



Legend to Fig. 2

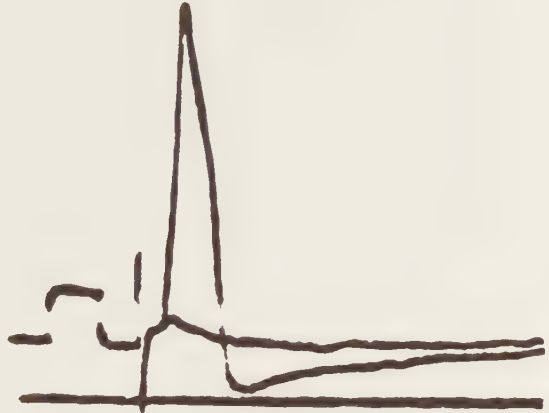
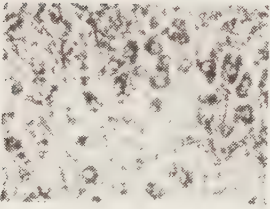
Morphological aspects of cell somas and axons of two distinct neuronal populations as seen in the center compartment and side compartment, respectively, of the compartmental cell culture system. Between 60,000 and 90,000 cells were obtained from chicken embryonic VSC or DRG as described previously (Sonderregger et al., 1983, 1984) and plated in the center compartment. After 3 days, the first axons, accompanied by some non-neuronal cells appeared in the two side compartments. However, the film of medium under the barrier between the center compartment and the side compartment was thin enough to prevent passage of neuronal cell bodies. After about one week in culture, the axons in the side compartments had grown together into thick fascicles and spanned at least 2/3 of the side compartment. Occasionally, axons reached the distal edge of the side compartment between day 8 and day 10 of culture.

a,c) Phase contrast photomicrographs of VSC and DRG cell somas, respectively, grown in the center compartment for 10 days. x 360.

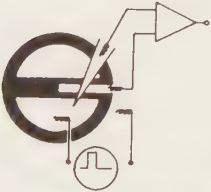
b,d) Phase contrast micrographs of the axonal fascicles of VSC and DRG neurons, respectively, as found in the side compartments after 1 week in culture, x 220.

neous activity among VSC cells was recorded. Extra-cellular stimulation of DRG neurons resulted in short, constant latency postsynaptic potentials in the VSC neurons that represented monosynaptic connections from DRG neurons to VSC neurons (Fig. 3b). Other VSC neurons showed responses

a



b



Legend to Fig. 3

Electrophysiological recordings demonstrating action potentials of DRG neurons and synaptic connections between DRG axons and VSC neurons. For electrophysiological recordings, the cultures were placed onto the stage of an inverted microscope (Zeiss) equipped with phase contrast optics. Intracellular recordings were made with glass microelectrodes, filled with 3 M KCl (resistance 120 to 150 Megohms), and potential signals were simultaneously displayed on an oscilloscope and chart recorder. Current injections were made through a bridge circuit. Extracellular stimulation of the presynaptic DRG neurons was obtained by pulses of 500 usec duration from a stimulus isolation unit applied between the center compartment and one of the side compartments. The stimulation threshold, determined by recording intracellularly from the cell somas in the center compartment, was 0.4 to 0.8 V of either polarity. The assay for synaptic connections was done by intracellular recording from the VSC neurons in the side compartments.

a) Intracellular recording from the soma of a DRG neuron during extracellular stimulation of the DRG axons extending from the center compartment to the side compartment. The stimulation and recording situation is illustrated in the inset. The upper oscilloscope trace shows a somal action potential as evoked by extracellular stimulation of the DRG axon. The lower trace probably represents an axonal action potential, which has been electrotonically conducted to the soma. Calibration pulse 10 mV per msec, extracellular stimulation pulse 1 V per 0.5 msec.

b,c) Extracellular stimulation of the presynaptic DRG axons and intracellular recording of postsynaptic potentials from VSC neurons, as schematically represented in the inset. The oscilloscope traces show an intracellular recording from a VSC neuron in a side compartment during repetitive extracellular stimulations of DRG axons. Short and constant latency responses can be seen as well as long and variable latency responses. Calibration pulse 5 mV for 5 msec. Extracellular stimulations (1 V for 0.5 msec) were immediately followed by a pulse of approximately 0.5 V for 0.5 msec to shorten the transient on the recording trace.

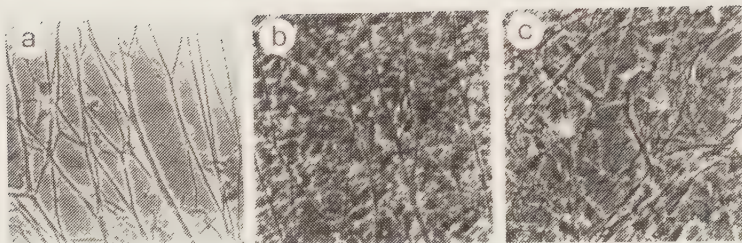
of longer and variable delay characteristic of polysynaptic responses. In areas where dense DRG processes entered the side compartment from the center compartment, most of the impaled VSC neurons showed mono-synaptic responses on extracellular stimulation of the DRG neurons. No stimulation-induced postsynaptic potentials were observed if the intracellular recordings were done in an area where no DRG axons could be seen emerging from beneath the barrier. This indicates that the stimulation of DRG axons was necessary to evoke the registered postsynaptic potentials and that

antidromic stimulation of VSC neurons by axons reaching into the center compartment was not important in the generation of these synaptic responses.

3.3. The pattern of fasciculation of DRG axons is specifically modulated by co-cultured glia.

To evaluate the influences of variations in the axons' glial environment, non-neuronal cells from the peripheral and central nervous system were locally co-cultured with the axons. Cells to be co-cultured were added one week after the dissociated DRG cells were plated in the center compartment. At a plating density of 15,000 cells per side compartment, the co-cultured cells reached confluency at about day 2 by spreading underneath the already present DRG axons. Hence, DRG axons were easily accessible for phase optical inspection at all times of the co-culture.

Light microscopic inspection after 3 days of co-culture revealed that co-culture with central neuroglial cells (Fig. 4c) led to a distinctly finer branching pattern of the axons with considerably less extensive fasciculation than observed in the axons without co-cultured cells (Fig. 4a) or co-cultured with peripheral glia (Fig. 4b). The degree of fasciculation has been found to depend on the strength of the adhesive forces acting among axons relative to the adhesion between axons and the environmental surfaces (Rutishauser et al., 1978). The observed fasciculation pattern thus suggests that under co-culture with central neuroglial cells adhesion with the surface is favored in comparison to the conditions where DRG axons are grown without any co-cultured cells or when peripheral neuroglial cells are co-cultured.



Legend to Fig. 4

Morphological aspects of axons of DRG neurons grown in different environments (phase-contrast optics, bars = 50 μ m).

Peripheral glial cells from DRG were obtained essentially as described previously (Wood, 1976) by growing undissociated DRGs in collagen-covered plastic dishes in the presence of nerve growth factor. After approximately one week in culture, the outgrowing axons were surrounded by a dense layer of non-neuronal cells that had migrated out of the ganglion, whereas the neuronal cell somas were still aggregated. To obtain a neuron-free population of cells, the aggregates of neuronal somas were cut out. The non-neuronal cells left behind were enzymatically dissociated, counted and added to the axons in the side compartments (approximately 15,000 cells per side compartment). Four days after plating for co-cultures, these cells were composed of about 70 - 80% Schwann cells as assessed by fluorescent labeling of S-100 protein (Sonderegger et al., 1985).

Central glial cells were obtained by cultivating dissociated spinal cord cells from 12 day old chicken embryos for about one week in the same medium that was used for the neuronal and the peripheral glial cells, except that for the first 3 days, the horse serum was replaced by fetal bovine serum. For co-culture with DRG axons, the cells were dissociated, counted and plated into the side compartment. Four days after initiation of the co-culture, these cells were composed of about 60% astrocytes and 15% oligodendrocytes as assessed by fluorescent marker studies using antibodies against glial fibrillary acidic protein and the cell surface antigens A2B5, O1 and O4 (Sonderegger et al., 1985). Virtually no neurons were present.

a) Axon fascicles in the side compartment of the compartmental cell culture system, 10 days after plating the dissociated DRG cells in the center compartment.

b) Co-cultured peripheral non-neuronal cells in the side compartments together with the axons of the DRG neurons.

c) Co-cultured non-neuronal cells form spinal cord in the side compartments.

4. THE COMPARTMENTAL CELL CULTURE SYSTEM AS A TOOL FOR THE STUDY OF THE EXPRESSION OF AXONAL PROTEINS

The separate access in the compartmental cell culture system to neuronal cell somas or axons and the fact that the thin film of medium connecting the compartments allows only very slow exchange of medium components can be exploited to control the environmental conditions of neuronal cell somas and their axons separately. Furthermore, since all neuronal proteins are thought to be synthesized by polysomes located in the cell soma and all axonal proteins are thought to reach their final location by axonal transport, this compartmental cell culture system offers an excellent tool for obtaining a selective representation of axonally transported proteins.

4.1. Selective analysis of the expression of axonal proteins.

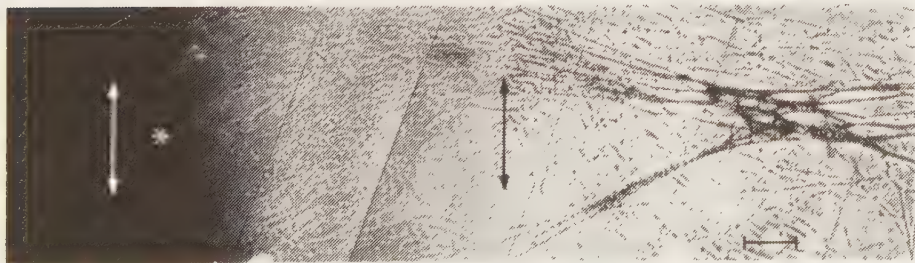
In our experiments on DRG and VSC neurons cultured in the compartmental cell culture system (Sonderegger *et al.*, 1983,1984) the newly synthesized proteins of the cells of the center compartment were metabolically labeled by addition of labeling medium containing methionine-free growth medium substituted with 15 μ M unlabeled methionine, and 1 mCi/ml ^{35}S methionine (approximately 1,000 Ci/mmol). The side compartments received the same medium, except that 4 mM unlabeled methionine replaced the radioactive methionine. Incubations of 40 h were used to allow for accumulation of the proteins of all axonal transport rate classes (Wilson & Stone, 1979). After labeling, 50 μ l of medium from each compartment was aspirated, protein precipitated by trichloroacetic acid, and the free ^{35}S methionine remaining in solution was counted with a beta-scintillation

counter at a counting efficiency of approximately 70%. This procedure served to provide an estimate of the leakage of radioactive label into the side compartment (for further discussion see below). After the remainder of the supernatant medium had been removed, the axons in the side compartments were washed twice with Dulbecco's PBS. The cellular material was dissolved in 2% sodium dodecylsulfate and 5% beta-mercaptoethanol at a temperature of 90°C, collected, pooled, and processed for analysis by two-dimensional electrophoresis. Two-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (two-dimensional SDS-PAGE) was done essentially as developed by O'Farrell (1975).

The compartments of the multicompartment cell culture system are connected to each other by a thin film of medium. It is critical for the success of these experiments that only those plates without bulk flow between the compartments are used for experiments. However, even in plates where no bulk flow of medium between the compartments occurred, contamination of the axonal proteins might result from the migration of heavily labeled non-neuronal cells from the center compartment to the side compartment during the 40-h period of labeling or from diffusion of radioactive amino acid through the film of medium between the compartments.

Migration of non-neuronal cells through the film of medium separating the compartments proved to be a slow process that appeared to stop after a few days in culture under the experimental conditions employed. The appearance of non-neuronal cells in the side compartments was usually first observed at 3 days, and never before 48 h, after plating. Taking into account a short lag phase after plating, we estimate the time for the passage of a cell through the

film of medium to be somewhat around 48 h. After a few days in culture, the appearance of new non-neuronal cells in the side compartments virtually ceased, possibly due to blockade of the medium passage by sessile cells. Indeed, when non-neuronal cells from spinal cord or DRG were grown in the center compartment in the absence of neurons, and when labeling was done according to the standard procedure, the radioactivity incorporated into the proteins of the side compartment cells was approximately the same as when the side compartment cells were exposed to side compartment medium containing diffused radioactive label from a previous labeling procedure. Radioautography of the area separating the center and side compartments, after removal of the Teflon inset (Fig. 5), showed a diffuse grain density occurring only in a zone immediately adjacent to the heavily labeled center compartment. Approximately two thirds of the separating area was essentially free of silver grains. In the side compartment, radioautographic label was confined to axon fascicles. These data strongly suggest that migration of labeled non-neuronal cells from the center compartment during the period of labeling does not contribute significantly to the radioactivity found in proteins



Legend to Fig. 5

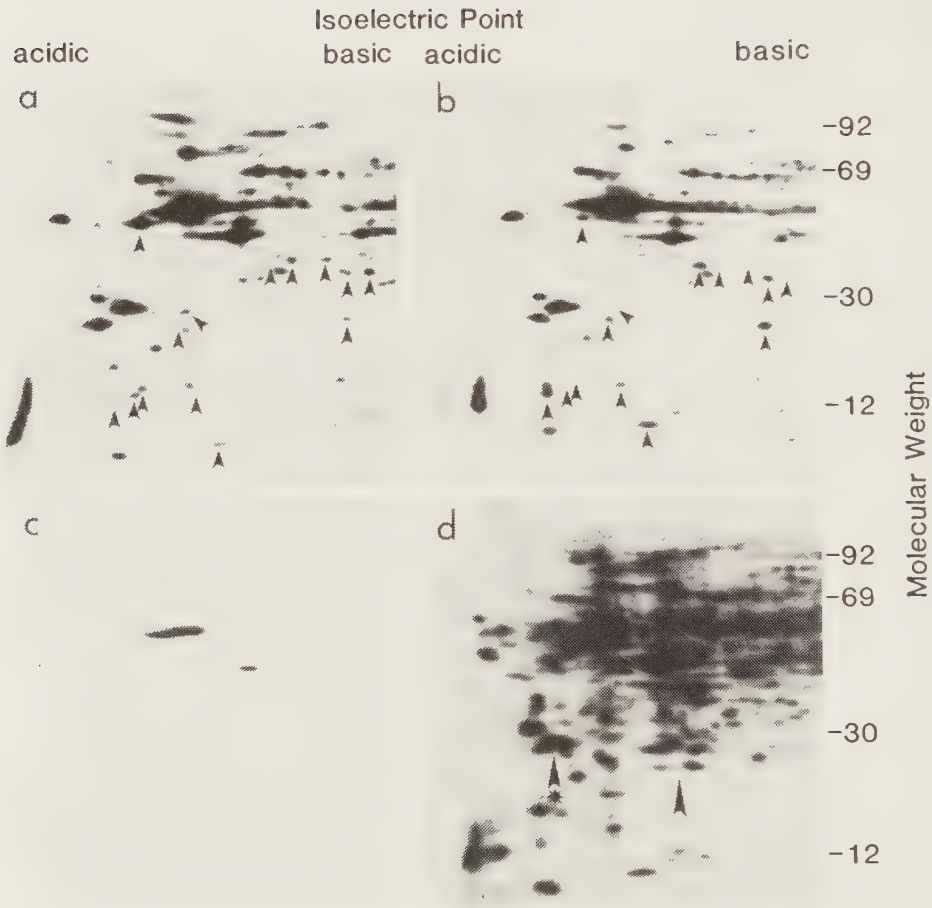
Radioautography of cells grown in the compartmental cell- culture system. Standard compartmental cell cultures were labeled by addition of ^{35}S methionine to the center compartment under conditons described previously. After incubation for 40 h the Teflon inset was carefully removed and the plates were processed for radioautography (for details cf. Sonderegger et al., 1984). After development, the radioautograms were photographed with phase contrast optics. Silver grains are seen over cells of the center, labeling, compartment, over a narrow zone (asterisk) in the separating area adjacent to the center compartment, and over the axon fascicles in the side compartment. Bar 0.1 mm. x 90. The arrows indicate the area of the film of medium separating the center compartment from the side compartment. Phase contrast optics.

of the side compartment. Radiograms were also prepared from cultures that, after labeling, were subjected to the standard collection procedure. After sodium dodecylsulfate/-mercaptoethanol dissolution of the cellular material of the side compartments, a broad band of non-neuronal cells of the area separating the center and the side compartments remained intact. Hence, the cells in the central part of this area were neither labeled by radioactive amino acid from the center compartment nor accessible to the sodium dodecylsulfate/-mercaptoethanol solution from the side compartment.

Some diffusion of radioactive amino acid through the film of medium was expected to occur and was considered a possible source of contamination of the axonally transported neuronal proteins by proteins incorporated by cells of the side compartments. Indeed, a minimal concentration of radioactive label (approximately 20 uCi/ml) was detected in the medium of the side compartments following an incubation period of 40 h, when a concentration of 1 mCi/ml ^{35}S methionine was used in the center compartment. The incorporation of radioactive methionine into proteins synthesized by

non-neuronal cells of the side compartments was competitively reduced by the addition of excess unlabeled methionine (4 mM) to the medium of the side compartments during the period of labeling. The degree of contamination was determined by collecting medium from the side compartments of five plates after a 40-h labeling period and adding it for 40 h to the side compartments of the same number of fresh plates containing approximately the same number of axons and accompanying non-neuronal cells. The incorporation of radioactivity into trichloroacetic acid precipitable material was found to be approximately 2% of that obtained by the usual labeling procedure and did not evoke any spots of labeled proteins other than those for tubulin and actin, the most abundant cellular proteins, when subjected to 2-dimensional SDS-PAGE (Fig. 6c).

This system therefore ensures that all the labeled proteins examined are derived from neurons, since these are the only cells whose cell bodies are in the central (labeling) compartment and whose axons are in the side (sampling) compartment. Contamination by labeled proteins from the inevitable, and also variable, non-neuronal components of the cultured tissue is virtually excluded. However, no claim can be made as to the completeness of the representation of the axonal proteins: 1) Higher resolution techniques may permit detection of many more proteins on a single gel; 2) the use of only one amino acid for labeling certainly excludes certain proteins from being visualized and 3) possible synthesis of proteins within axons (Edstrom & Sjostrand, 1969; Bondy & Purdy, 1975; Black & Lasek, 1977; Frankel & Koenig, 1978; Koenig, 1979) may be dependent on the local availability of necessary amino acids.



Legend to Fig. 6

Two-dimensional electropherograms of the axonal proteins of VSC and DRG neurons (all panels 60% reduced). Samples were matched for trichloroacetic acid-precipitable radioactivity (approx. 400,000 cpm/gel). Elec-

trophoresis was done as described (O'Farrell, 1975). Subsequently, the gels were dried and fluorography was done according to the principles developed by Bonner and Laskey (1974).

a,b) Protein patterns of VSC neurons and DRG neurons, respectively. Each protein present in markedly different quantity in VSC axons and DRG axons is indicated by an arrowhead. The extrapolated location, relative to the surrounding spots, of absent or barely visible proteins, is indicated.

c) Control for contamination of the axonal proteins by labeled proteins derived from non-neuronal cells of the side compartment. After a labeling period of 40 h, the side compartment medium from five plates was collected and transferred to the side compartment medium of five fresh plates containing an equivalent number of axons and non-neuronal cells.

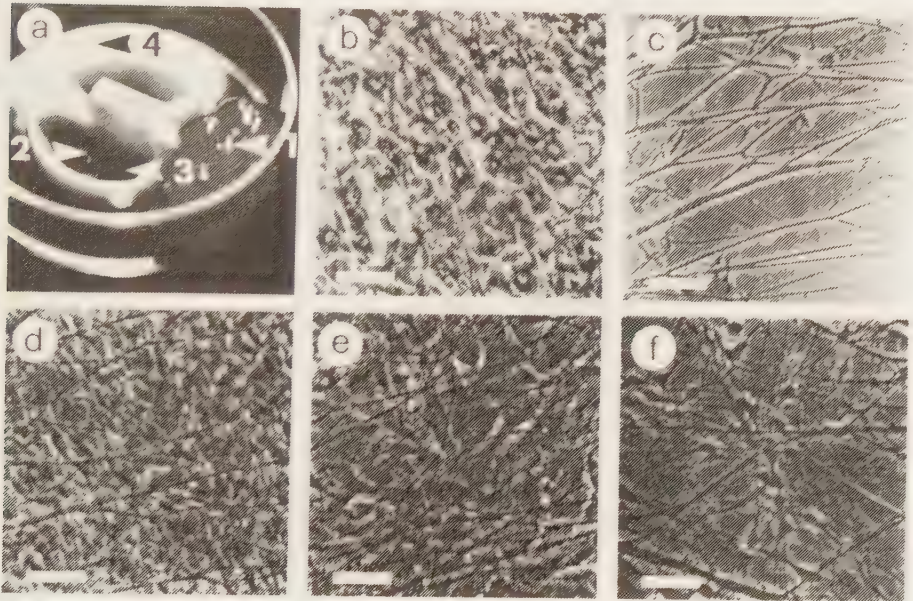
d) Protein pattern of cellular material of the center compartment of a DRG culture. A cluster of proteins that is absent from the axonal proteins pattern is indicated by an arrowhead. The "landmark" spot used for approximated visual matching of the density of the three protein patterns is indicated by an arrowhead with an asterisk.

4.2. The study of local axon-glia and axon-neuron interactions.

A feature of the compartmental cell culture system is that it allows to variation of the cellular and humoral environment of axons while keeping the cellular and humoral environment of the cell somas constant. Hence, this experimental system offers an experimental approach for the study of local interactions between axons and co-cultured glia or neuronal target cells. In our studies on the environmental modulation of the expression of axonal proteins, glia cells or potential neuronal target cells were co-cultured locally with the axons extending to the side compartments at a point in time when the axons spanned more than 2/3 of the width of the side compartment (day 7). At this time, the DRG cells of the center compartment (neurons plus non-neuronal cells) had reassociated and resumed a histotypic pattern for DRGs.

As previously mentioned (cf. 4.1), diffusion of radioactive amino acids from the center (labeling) compartment to the side compartments has to be considered as a feature inherent to the system. As a consequence, cells present in the side compartments had to be expected to incorporate radioactive label into their proteins. When labeling was done with only a few non-neuronal cells in the side compartments, the radioactive amino-acid that accumulated in the side compartment medium during the 40 h period of labeling was too low to contribute significantly to the radioactively labeled proteins found in the side compartments (cf. 4.1.). However, when the axons of the side compartments were co-cultured with high numbers of glial or other cells, the contribution of locally synthesized proteins became significant. Hence, two additional measures of precaution were necessary in order to obtain contamination-free representation of axonal proteins of DRG neurons in the presence of co-cultured cells. The volume of the nonradioactive medium in the side compartments had to be increased in order to dilute the radioactive amino acid diffusing through the film of medium from the center compartment, and the number of co-cultured cells had to be reduced. The volume of medium in the side compartment during the time of labeling was increased by cutting the outer rim of the standard Teflon inset to about $1/3$ of its original height, except in the area delineating the center compartment (Fig. 7a). For labeling, the opening of the center compartment was sealed with a grease plug (Fig. 7a, arrowhead 2), and medium was added until the fluid of the side compartment and the rest of the dish (Fig. 7a, arrowhead 1) were confluent. This increased the side compartment volume from about 0.5 to 3.5 ml. The number of cells used for co-culture with the axons of the side compartments was reduced without changing the cell density by placing a grease wall in the middle of the side compartment (Fig. 7a, arrowhead

3). Cells to be co-cultured were plated only in the area adjacent to the barrier, where axons grow in from the center compartment (Fig. 7a, arrowhead 4).



Legend to Fig. 7

Morphological aspects of axons of DRG neurons grown in different environments. DRG cells from 10 day old chicken embryos were cultured in the compartmental cell culture system. After 10 days in culture, the neurons and accompanying non-neuronal cells of the center compartment had re-associated in a histiotypic fashion, whereas the axons in the side compartment had extended and associated into fascicles. Both peripheral non-neuronal cells, obtained from DRGs, and central non-neuronal cells, obtained from spinal cord, were then co-cultured with the axons.

a) The compartmental cell culture system as modified for metabolic labeling of axonal proteins in the presence of co-cultured cells in the side compartment and continuous conditioning studies. The outer rim of the Teflon inset was cut to about one third of the original height, except in the delineation of the center compartment.

- Arrowhead 1: Grease plug to separate center compartment from the joint outer compartment during the period of labeling.
- Arrowhead 2: Grease wall across the side compartment to reduce plating area of co-cultured cells of the side compartments.
- Arrowhead 3: Area of co-culture in the side chamber for direct cell contact between axons and co-cultured cells.
- Arrowhead 4: Area of co-culture outside the Teflon inset for studies of humoral interaction between axons and co-cultured cells.

b) Somas of DRG neurons and glia in the center compartment (Phase-contrast optics).

c) Axon fascicles in the side compartment of the compartmental cell culture system, 10 days after plating the dissociated DRG cells in the center compartment (Phase-contrast optics).

d) Co-cultured peripheral glial cells in the side compartments together with the axons of the DRG neurons. At the time in culture corresponding to the middle of the metabolic labeling period, the peripheral non-neuronal cells were composed of about 70-80% of Schwann cells, as assessed by fluorescent labeling of S-100 protein. Virtually no neurons were present (Sonderegger et al., 1985).

e) Co-cultured central glial cells from spinal cord in the side compartments together with axons of the DRG neurons. Central non-neuronal cells, at the time in culture corresponding to the middle of the metabolic labeling period, were composed of about 60% astrocytes and 15% oligodendrocytes as assessed by fluorescent marker studies using antibodies against glial fibrillary acidic protein and the cell surface antigens A2B5, O1 and O4 (Sonderegger et al., 1985). Virtually no neurons were present.

f) Co-cultured ventral spinal cord cells in the side compartment together with axons of the DRG neurons. Synapse formation between DRG axons and ventral spinal cord neurons in this system has previously been reported (Sonderegger et al., 1983).

Control for contamination of the axonal proteins by labeled proteins derived from non-neuronal cells of the side compartment was accomplished as described for the situation without co-cultured cells (cf. 4.1.). The side compartment medium from five plates was collected after a labeling period of 40 h and transferred to the side compartment of five fresh plates containing an equivalent number of axons and non-neuronal cells. After incubation for 40 h, the radioactivity incorporated into trichloroacetic acid-precipitable cellular material of the side compartments was

approximately 1% of that found when axonal proteins were labeled by addition of the radioactive amino acid to the medium of the center compartment. When this material was subjected to 2-dimensional SDS-PAGE and exposed for fluorography under standard conditions, no spots of labeled proteins other than tubulin and actin were observed in the two-dimensional electropherogram. Hence, by providing some measures for diminishing contamination by non-neuronal cells selective analysis of axonal proteins can be accomplished from axons that are embedded in dense monolayers of non-neuronal cells.

4.3. The compartmental cell culture system as a tool for continued conditioning in studies of humoral glia-derived factors on axonal behavior and protein expression.

The modified version of the compartmental cell culture system (cf. 4.2) offers an elegant method for the investigation of axonal behavior and the expression of axonal proteins under the influence of soluble cell-derived factors. For this purpose, the cells under consideration for a possible humoral interaction with axons are cultured outside the rim of the side compartment (Fig. 7a, arrowhead 4). After the co-cultured cells have adhered to the plate additional medium is added so that the medium of the side compartment (containing axons) and the medium of the areas outside the Teflon inset (containing test-cells) become confluent. Under these conditions, the axons and the test-cells are separate from each other but share the same medium and, hence, may humorally interact. This experimental set-up may be especially suitable for the analysis of relatively short lived secreted substances, since constant supply from the co-cultured cells is provided.

4.4. Unequivocal identification of proteins secreted from axons of distinct neuronal subpopulations.

The contribution of neurons or their products to the interactive pattern of the nervous system has been corroborated over the past years. In particular, axons, besides their signal conducting function, have been found to have trophic influences on surrounding glia and target neurons. Part of such influences may be implemented by secretion of proteins from axons. Indeed, in addition to putative transmitter peptides, a number of proteins have been found to be secreted by axons. One of these proteins was functionally identified as a plasminogen activator and is thought to be important in neuron migration and axon elongation (Krystosek & Seeds, 1981a, 1981b). A number of axonally secreted proteins, with not yet determined function, are intriguing by the fact that they occur specifically in subpopulations of sympathetic neurons (Sweadner, 1981). Since axonally transported proteins include proteins destined to be secreted, metabolic labeling, collection and analysis of the axon-conditioned medium of the side compartment offers a unique way to prove the axonal origin of such secreted proteins. This is especially true for neuronal subpopulations that cannot be cultured completely free of accompanying non-neuronal cells, a fact that holds true for most neuronal tissue culture systems. (Fischbach & Nelson, 1977).

5. EXAMPLES OF APPLICATION IN THE SEARCH FOR PROTEINS WITH A ROLE IN AXON DEVELOPMENT OR REGENERATION.

5.1. Protein differences between axonal subpopulations may point to proteins involved in subpopulation-specific functions.

The specific macromolecular content of each neuron or group of neurons may specify its behavior in terms of axonal functions (Sperry, 1963; Gottlieb & Glaser, 1980), and thus direct the topographic arrangement and the formation of specifically targeted connections of cells within the nervous system. The distinctive identity of individual classes of neurons has been well defined electrophysiologically, morphologically, and with respect to presumptive neurotransmitters. Furthermore, there is evidence for the involvement of particular features of the axonal surface in axonal fasciculation (Bray et al., 1980; Raper et al., 1983a; Rutishauser et al., 1978), pathfinding (Raper et al., 1983b), and specific synapse formation (Fuchs et al., 1981). However, the molecular structures that distinguish subpopulations of neurons from each other and thus determine their distinct behavior, are poorly understood. The probes for macromolecular differences between nerve cells include the binding of antibodies (Fields et al., 1978; Vulliamy et al., 1981; Cohen & Selvendran, 1981; Omlin et al., 1984) or lectins (Hatten & Sidman, 1977; Hatten et al., 1979; Pfenninger & Maylie-Pfenninger, 1981; Schwab & Landis, 1981; Zurn, 1982) and the detection of transmitter-related enzymes (Wilson et al., 1972; Altschuler et al., 1982) or other proteins, e.g. the calcium-binding protein parvalbumin (Celio and Heizmann, 1981). In recent studies cellular and extracellular proteins expressed with different abundance in cultured sympathetic neurons expressing

adrenergic or cholinergic phenotypes have been identified (Sweadner, 1981; Braun et al., 1981). To address the question of whether differences in the expression of axonal proteins of two different neuronal populations could be resolved, we have grown two distinct, although not homogeneous, populations of neurons, derived from dorsal root ganglia and spinal cord, respectively, in the compartmental cell culture system (Fig. 1 & 2). Both populations of neurons have been studied extensively. When grown in ordinary cell culture systems both populations retain distinct features characteristic for their in vivo counterparts, such as their axonal and dendritic morphology, typical shape of their action potentials as pharmacological responses, and establishment of synapses with their natural target cells (for review cf. Fischbach & Nelson, 1977).

The cell culture conditions were designed to compare two populations of neurons at a time of active axon outgrowth in vitro. Additionally, the cells to be cultured were dissected at an embryonic stage at which active axon outgrowth was also known to occur in vivo. Most of the neuronal cells destined for the ventral horn withdraw from the mitotic cycle in the developing chick embryo during the third day (stages 17-18 of Hamburger and Hamilton; Hamburger & Hamilton, 1951; Hollyday & Hamburger, 1977). The birthdate of the bulk of the neuronal cells of the DRG, however, has been determined to occur between embryonic day 4.5 and 7.5 (McMillan Carr & Simpson, 1978). Hence the VSC and the DRG were dissected on about the third and fourth day, respectively, after the majority of their neurons had entered the postmitotic phase and at a time of active axon outgrowth and synapse formation (Stelzner et al., 1973). The two neuronal populations used in the present study were, therefore, developmentally about equivalent at the time they were placed in culture.

The two-dimensional electrophoretic representations of the axonal protein patterns of VSC and DRG neurons are quite similar (Fig. 6a and b, respectively). However, distinct, and in some cases qualitative differences have been revealed. In three independent experiments we found seven proteins in the molecular weight range between 10,000 and 100,000 (where the major part of the cellular proteins is usually found) that were more abundantly expressed in VSC axons than in DRG axons. Conversely, seven other proteins in the same molecular weight range were expressed primarily in DRG axons. It must be pointed out that these data do not represent comparisons of absolute amounts of axonal proteins, but rather comparisons of the relative amounts of proteins that were produced and axonally transported during a 40 hour period of labeling. The ratios of the absolute amounts of a particular protein would be expected to be a complex function of synthesis, axonal transport, post-translational modification and degradation. The gel pattern of cellular material from the center compartment was, as anticipated, substantially more complex than that of the axons (Fig. 6d). Matching an arbitrary landmark spot for equal density in both gels (Fig. 6d, arrowhead with asterisk) revealed many more proteins in the cellular protein pattern than in the axonal protein pattern. Sets of proteins not visible in the axonal protein pattern were evident (Fig. 6d, arrowhead), and some areas were crowded with spots close to confluency. It is impossible, however, to assign any of these proteins to a particular cell type or to a cellular compartment due to the complex composition of the cellular material.

The high degree of similarity observed between the axonal protein patterns of VSC and DRG certainly reflects the commonalities of axons in these neuronal subsets. However, as stated previously, there are a number of morphological

and functional characteristics that clearly distinguish axons of these different neuronal populations from each other. The axonal proteins observed to differ in their expression in these two enriched neuron populations are likely candidates for a role in the implementation of those specific functions that distinguish VSC and DRG axons from each other.

5.2. The expression of a few axonal proteins is differentially modulated by non-neuronal cells of the peripheral and central nervous system.

Peripheral glia have long been recognized to provide a more favorable environment for axon regeneration than central glia (Ramon y Cajal, 1928). Cajal's finding is impressively corroborated by more recent experiments showing that peripheral nerve segments grafted into mammalian central nervous system were able to support re-outgrowth of axons from central neurons markedly better than the normally present central glial cells (Sugar & Gerard, 1940; Kao et al., 1977; Richardson et al., 1980; David & Aguayo, 1981). The virtual absence of structural and functional repair in the central nervous system is thought to depend on the impeding action of the central glia on the re-outgrowth of axons. However, the issue of whether the adverse action of central glia is due to a mechanical barrier of scar tissue or to environmentally induced changes in the intrinsic capability of neurons to regenerate their axons remains unsettled (Reier et al., 1983).

Dorsal root ganglion neurons have been recognized in a variety of studies to respond readily to environmental signals. Axons growing out from the neuronal somas in dorsal root ganglia are first entirely surrounded by a peripheral glial environment and form one single fascicle,

termed dorsal root. Upon entering the spinal cord at the transitional fringe, they come into contact with central glia and split to reach their various destinations. Upon meeting spinal cord neurons, they decide whether or not to stop growing and make a synapse. Obviously, there is a varying set of functional features that axons must be endowed with in order to compensate for the varying requirements they encounter on their path from the ganglion to their target neurons. Many of their functional capabilities are silent and, when needed, are expressed in response to environmental cues. After injury of dorsal roots, the axons of DRG neurons regenerate readily as long as they are in a peripheral glial environment but virtually stop elongation at the transition from the peripheral to the central nervous system (Perkins, 1980; Stensaas et al., 1979; Reier et al., 1983). In the case of cultured DRG neurons, environmental influences have been found to act on axon elongation (Levi-Montalcini, 1954; Baron van Evercooren et al., 1982; Edgar et al., 1984), the guidance of the axonal pathway (Letourneau, 1975; Gundersen & Barret, 1979), the choice of the neurotransmitter (Mudge, 1981) and the morphology of the axons' initial segment (Mudge, 1984). These facts make DRG neurons a particularly attractive model for the study of the environmental regulation of axonal functions and the intriguing behavioral differences of axons during development and repair from injury in the central and peripheral nervous system of amniotic vertebrates.

As a first step towards the elucidation of the modulatory influences exert by peripheral and central glia upon axons of DRGs, we examined whether the protein composition of axons was subject to environmentally induced changes when the axons were locally co-cultured with peripheral and central glia (Fig. 7). Both peripheral non-neuronal cells, obtained from DRGs, and central non-neuronal cells, ob-

tained from spinal cord, were then co-cultured with the axons (Fig. 7d and 7e, respectively). The cellular and humoral environment around the DRG cell somas and the proximal area of the axons was kept constant. The newly synthesized proteins were then metabolically labeled with ^{35}S methionine added to the neuronal somas in the center compartment. After incubation for 40 h, the cellular material of the side (axonal) compartments was harvested. The side compartment proteins were then analyzed by two-dimensional polyacrylamide gel electrophoresis (O'Farrel, 1975) followed by fluorography (Bonner & Laskey, 1974). Axonal proteins that were synthesized and axonally transported when the axons were in contact with either peripheral (DRGNN) or central (SCNN) non-neuronal cells were quantified and compared with the proteins that were synthesized when the axons were grown without any co-cultured cells, using GELLAB, a system for computerized gel image analysis (Lipkin & Lemkin, 1980; Lemkin *et al.*, 1982, 1984; Lemkin & Lipkin, 1983a,b).

Under the conditions employed for these experiments, more than 400 proteins were discernible on each gel. Computerized quantitation of the individual protein spots using GELLAB revealed that the bulk of the axonal proteins were expressed at identical relative quantities under all three experimental conditions. This finding was not surprising, since the constancy of the gross morphology (with exception of the fasciculation pattern) caused us to expect constancy of the bulk of the structural proteins, which comprise a substantial proportion of the major cellular proteins.

Twelve proteins, out of a total of more than 400 discernible spots, were found to differ in at least one of the three possible comparisons (Table 1: DRGNN vs. none, SCNN vs. none, SCNN vs. DRGNN). These modulated proteins were

Table I

Environmental effects on the expression of axonal proteins: Different modulatory effects of co-cultured peripheral and central non-neuronal cells.

Subset	Spot no.	Co-cultures compared (Ratio of mean normalized densities)		
		DRGNN vs. none	SCNN vs. none	SCNN vs. DRGNN
I	435	0.51 ^Δ	0.38 ^Δ	(0.74)
I	582	0.29*	0.21*	(0.73)
(I)	335	0.60*	(0.65)	(1.08)
(I)	516	0.61*	(0.63)	(1.04)
II	86	3.83*	7.21*	1.89 ^Δ
(II)	85	(0.59)	(3.69)	6.67 ^Δ
(II)	90	(1.76)	(0.73)	0.42*
(II)	484	(1.30)	(0.63)	0.48 ^Δ
III	162	1.84*	(1.16)	0.63*
(III)	359	(1.79)	(.90)	0.51*
IV	361	(0.82)	0.32 ^Δ	0.39 ^Δ
IV	462	(1.14)	0.17 ^Δ	0.15*

Legend to Table I

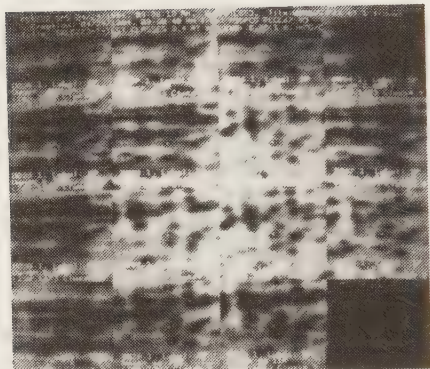
Ratios of the mean normalized density values of corresponding proteins that have been found significantly different in at least one of the comparisons of the three experimental classes of co-culture, namely DRG axons without co-cultured cells (none), DRG axons with co-cultured peripheral glial cells (DRGNN), and DRG axons with co-cultured central glial cells (SCNN).

Quantification of the individual proteins was done with GELLAB (Lipkin & Lemkin, 1980; Lemkin et al., 1982,1984; Lemkin & Lipkin, 1983a,-1983b). The fluorographic replicas of the two-dimensional electropherograms are scanned along with a neutral density calibration wedge, by a high resolution black and white TV camera and digitized into 512x512 picture element images with grey values (white to black). The images are then calibrated in terms of optical density units and thereafter all measurements are in terms of integrated optical density. GELLAB successively segments individual spots in a gel image, pairs corresponding spots from different gels and finally merges corresponding spots into a so-called composite gel data base. The user selects a reference gel (denoted the Rgel) and interactively defines a small number of corresponding landmark spots between the Rgel and each of the remaining N-1 gels of the Rgel. Corresponding spots in the composite data base are called Rspot sets and consist of those spots in the N gels which correspond to the same spot in the Rgel. The data were normalized by the least square method (Lemkin et al., 1983a). The N gels were subdivided into groups of gels which included gels of the same experimental condition. These groups are called experimental classes in GELLAB. Statistical comparisons were based on at least 5 gels per class obtained by independent experiments using F-tests at confidence limits of 0.90 and 0.95.

If a statistically significant difference of the normalized density values for a protein spot occurred between two experimental classes (F-test, $n = 5$) at a confidence limit of 0.95, the ratio value is marked by an asterisk (*). If a difference was revealed at a confidence limit of 0.90, the ratio value is labeled with a triangle (). If no difference was revealed by the statistical test, the ratio of the mean normalized densities of the compared experimental classes is put in parentheses. The environmentally modulated proteins were subdivided into 4 subsets, according to the pattern of differences observed in the comparisons of the 3 experimental classes: Proteins changed by both peripheral and central neuroglia to the same extent (subset I-proteins); proteins changed by peripheral and central neuroglia to a different extent and possibly in opposite directions (subset II); proteins changed specifically by peripheral neuroglia (subset III) and, and proteins changed specifically by central neuroglia (subset IV). Subset numbers in parentheses indicate proteins that could be assigned only tentatively to a particular subset because of absence of complementarity in the statistical comparison between the 3 experimental classes.

controlled for appropriate pairing of corresponding spots on different gels by generation of mosaic images (for an example cf. Fig. 8). These proteins were subdivided into 4 subsets according to the pattern of differences they showed. Proteins 435 and 582, and possibly 335 and 516, were changed under the influence of both peripheral and central non-neuronal cells to about the same extent (Table 1: subset I proteins; Fig. 9a). Protein 86, and possibly 85, 90 and 484, were changed by both peripheral and central non-neuronal cells, but the extents of the induced changes differed (Table 1: subset II; Fig. 9b). Protein 162, and possibly 359, were changed specifically by co-culture with peripheral, but not with central, non-neuronal cells (Table 1: subset III; Fig. 9c), whereas proteins 462 and 361 were specifically changed under the influence of central, but not peripheral, non-neuronal cells (Table 1: subset VI; Fig. 9d).

The changes in the synthesis of axonal proteins of DRG neurons thus include changes induced specifically by either peripheral or central non-neuronal cells as well as changes that are induced by the presence of both peripheral and central non-neuronal cells. The cell types responsible for the registered changes remain to be determined, since both the peripheral and the central non-neuronal cell populations used for co-culture with the axons are heterogeneous (Sonderegger et al. 1985). The observed differences in the pattern of axonal proteins might possibly be generated by mitotic expansion of a subset of neurons or by differential survival of neuronal or axonal subpopulations. However, the DRGs were dissected after the DRG neurons had become post-mitotic during embryonic development (McMillan Carr & Simpson, 1978) and DRG neurons of 10 day old chicken embryos have been shown to remain post-mitotic after they have been brought into tissue culture (Chalazonitis &

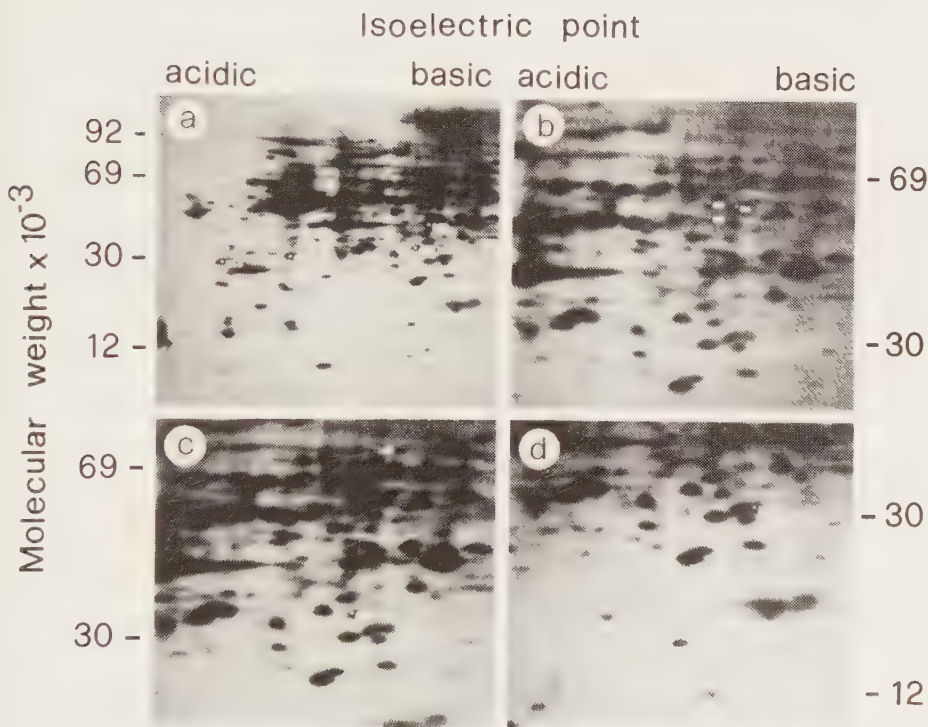


Legend to Fig. 8

Mosaic images as a tool for controlling appropriate pairing of corresponding spots of different gels.

The GELLAB system for the computerized analysis of 2-dimensional SDS-PAGE used to detect protein differences between axons exposed to different environmental conditions provides the facility to generate so-called mosaic images. A mosaic image is an image composed of panels of subregions of the gel images surrounding a particular spot ordered by increasing normalized density. The assumed relative coordinates of the center of the spot to be investigated are positioned in the center of each panel and labeled with a white dot. Each panel represents another gel and 16 gels can be displayed in one mosaic image. The spots of the surrounding region give a morphological context which allows verification that the spot indicated is in fact the one under investigation. As mispaired spots are easily detected by visual inspection in these mosaic images, this facility provides an excellent tool for the control of accurate spot pairing. We made extensive use of the mosaic image facility in checking for correct pairing of every spot that was found to be significantly changed in any of the pairwise comparisons between experimental classes.

As an illustration of the mosaic image facility of GELLAB which served an important role in the control for correct pairing of corresponding spots between gels, a mosaic of spot 85 is presented, comparing experimental clones 3 (SCNN) and 4 (DRGNN).



Legend to Fig. 9

Digitized images of 2-dimensional electropherograms indicating those proteins that are environmentally modulated by peripheral or central glia cells. The GELLAB system has facilities for visualizing selected protein spots in a copy of any of the original gel images (Lipkin & Lemkin, 1980; Lemkin et al., 1982, 1984; Lemkin & Lipkin, 1983a, b). The pictures were taken from a high resolution raster scan display by a polaroid camera. Picture b, c and d are photographs taken from 2 x 200 zoomed images focused at selected areas of the gel picture.

a) Proteins changed by both peripheral and central neuroglia to the same extent (subset I proteins).

- b) Proteins changed by both peripheral and central neuroglia but to a different extent and possibly in opposite direction (subset II).
- c) Proteins changed specifically by peripheral neuroglia (subset III).
- d) Proteins changed specifically by central neuroglia (subset IV).

Fischbach, 1980). Visual inspection did not reveal any signs of deteriorating or dead neuronal cell somas among the histiotypically organized tissue of the center compartment through the end of the experiments; nor was any morphological evidence for deterioration of axons observed. The amounts of radioactivity incorporated into the axonal proteins under all three experimental conditions were found to be in the same range. Thus, we consider the observed phenomenon most probably due to modulatory effects on the protein synthesis of a preexisting set of cells and their axons.

The observed data thus demonstrate that the local cellular environment of axons modifies their protein composition in a specific way. Thus, local signals impinging on neuronal axons must be capable of regulating biosynthetic activity and/or translocation mechanisms in the neuronal cell body or to activate local post-translational modifications in the axons. The signals could reside in the cell surface properties of the co-cultured non-neuronal cells or in the soluble substances they secrete. Modulation in the protein content of axons may represent the macromolecular correlate of modulation in some yet undetermined axonal function such as elongation, sprouting, fasciculation, transmitter synthesis or the capability for synapse formation.

5.3. Experimentally evoked modulation of axonal proteins as a tool for the identification of proteins involved in concomitantly modulated axonal functions.

As opposed to in vivo studies, one of the principal advantages of the compartmental cell culture system used here is that experimental variation of the local, cellular and humoral environment of axons can be accomplished virtually at discretion. To change the local axonal environment in order to produce changes in axonal functional properties and then demonstrate concomitantly altered protein expression may be a useful step towards relating proteins to particular axonal functions. The identification of environmentally modulated proteins and the comparison of their modulation profiles under a series of defined local environmental conditions is presented here as an approach to obtain some clues for the identification of the molecular correlates of axonal functions and their regulation.

As an example, a comparison of the expression of proteins of DRG axons under four different local axonal co-culture conditions, viz. axons grown without co-cultured cells, axons with co-cultured peripheral glia, axons with co-cultured central glia and axons with co-cultured spinal cord cells (glia plus neurons) is discussed here with some detail (Fig. 7). Under the conditions employed for these experiments, more than 400 protein spots were discernible on each gel. Computerized quantification of the individual protein spots with GELLAB (Lipkin & Lemkin, 1980; Lemkin & Lipkin, 1983b) revealed that the bulk of the axonal proteins were expressed at identical relative quantities under all three experimental conditions. Thirteen proteins were significantly changed in at least one of the 6 possible paired comparisons of the four experimental classes. The ratios of the mean least-squares normalized densities of these pairwise comparison ranged from 0.15 to 7.69 (Table II).

Table II
Environmental effects on the expression of axonal proteins: Modulatory effects of glia and target neurons.

Spot no.	M.W.	Co-cultures compared (ratio of mean normalized densities)							
		DRGNN vs. none	SCNN vs. DRGNN	VSC vs. SCNN	SCNN vs. none	VSC vs. none	VSC vs. DRGNN		
85	65K	(0.59)	6.67	(0.99)	(3.69)	3.70*	6.18*		
86	65K	3.83*	1.89	(1.09)	7.21*	7.69*	2.06*		
90	60K	(1.76)	0.42*	0.63	(0.73)	(0.46)	0.26*		
160	97K	(1.45)	0.56	(0.83)	(0.81)	(0.67)	0.46*		
162	97K	1.84*	0.63*	(1.34)	(1.16)	(1.55)	0.85*		
335	48K	0.60*	(1.08)	(0.98)	(0.65)	0.64*	(1.7)		
359	37K	(1.79)	0.51*	(0.80)	(0.90)	(0.72)	(0.40)		
361	35K	(0.82)	0.39	3.85	0.32	(1.23)	(1.49)		
435	37K	0.51	(0.74)	(1.79)	0.38	(0.68)	(1.33)		
462	13K	(1.14)	0.15*	4.76	0.17	(0.77)	(0.68)		
484	27K	(1.30)	0.48	(0.80)	(0.63)	0.50	0.39*		
516	31K	0.61*	(1.04)	(0.76)	(0.63)	0.48*	(0.79)		
582	34K	0.29*	(0.73)	(1.54)	0.21*	0.33*	(1.12)		

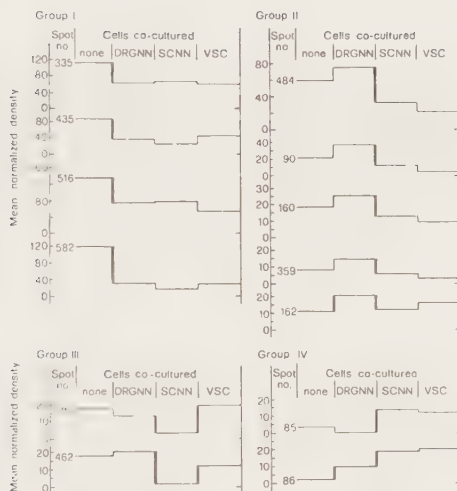
Legend to Table II

Ratios of the mean normalized density values of corresponding proteins that have been found significantly different in at least one of the comparisons of the four experimental classes of co-culture, namely DRG axons without co-cultured cells (none), DRG axons with co-cultured peripheral glial cells (DRGNN) and DRG axons with co-cultured central glial cells (SCNN) and DRG axons with co-cultured ventral spinal cord cells (VSC). If a statistically significant difference of the normalized density value for a protein spot occurred between two experimental classes (F-test, $n = 5$) at a confidence limit of 0.95, the ratio value is marked by an asterisk (*). If a difference was revealed at a confidence limit of 0.90, the ratio value is labelled by a triangle (). If no difference was revealed by the statistical test, the ratio of the mean normalized densities of the compared experimental classes is put in parentheses. Part of these data are taken from Table II. They are listed here for the sake of completeness.

Assuming that each protein that can be observed is related to some function, it is tempting to think of proteins modulated in their expression in terms of proteins forming building-blocks of axonal functions or their regulatory apparatus. Hence, plasticity in the expression of axonal proteins in response to different topographical provenance of the surrounding cells may reflect environmental modulation of axonal functions. In the regulation of gene expression, various levels of control have been recognized. As a consequence, if we think of axonal functions as the phenomenological product of the concerted operation of a smaller or larger number of molecules, their modulation may be operated at a variety of possible hierarchical levels and by a large number of possible mechanisms. Modulation of a function may be controlled not only by variation of a supply of the necessary molecular building-blocks, but also by their structural modification, which may occur during their synthesis, during axonal transport or at their axonal location.

To test whether the environmentally modulated proteins showed any common traits when their relative abundance under different environmental conditions was considered, histogram-type graphic representation was chosen and the mean normalized densities of these protein spots under the various experimental conditions were plotted (Fig. 10). As evident from Fig. 10, the modulated proteins roughly subdivided into 4 groups. Group I contains proteins characterized by high abundance in the absence of any co-cultured cells (see also Fig. 11a). Co-culture of any of the 3 populations reduced these proteins markedly. Group II contains 5 proteins, with high abundance under co-culture with periphal glia as the most striking feature (see also Fig. 11b). III is composed of 2 proteins that suffered a marked depression by co-culture with central glia (see also Fig. 11c). Group IV was composed of 2 proteins highly abundant in the case of co-culture with central glia and spinal cord cells, but otherwise did not behave similarly throughout the various conditions (see also Fig. 11d). Although exhibiting common traits, these groups seemed to be heterogeneous in other features. The need for a technique giving a quantitative estimate for the similarity of the clusters, as well as allowing definition of "stopping rules" for the clustering process, was obvious.

Consequently, the modulated proteins were subjected to cluster analysis - a technique designed to subdivide objects into groups containing members which are considered to be similar (in some abstract feature space) (Anderberg, 1973; Hartigan, 1975). In the present study, we used the ratios of the mean normalized densities in all permutations of paired comparisons of the experimental classes as the features for the clustering process. The data obtained clearly reveal close relationships between some of the manually grouped proteins and distinguish them from others



Legend to Fig. 10

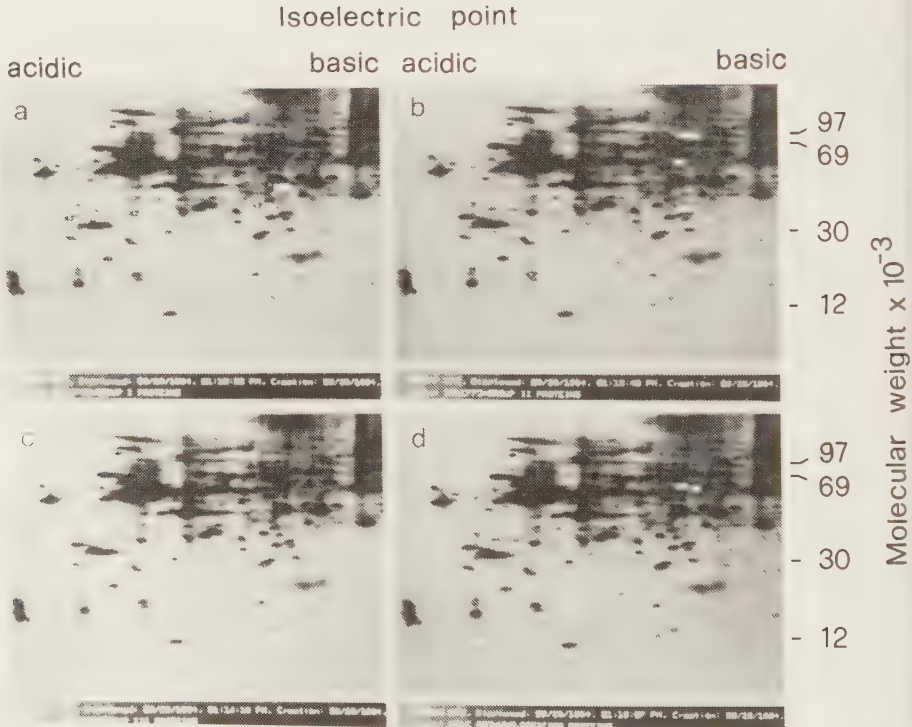
Modulation profiles of the 13 proteins previously identified to be modulated by environmental influences. Histogram-type plots of mean normalized density versus co-culture condition. The thick vertical bars indicate statistically significant differences in the comparison of the adjacent experimental classes. The modulation profiles were manually clustered according to their most prominent feature. Heterogeneity of these groups is obvious at more detailed inspection and has been dealt with by computerized cluster analysis.

Group I-proteins: Proteins with high relative abundance in the absence of any co-cultured cells (none).

Group II-proteins: Proteins with high relative abundance under local co-culture with peripheral glia (DRGNN).

Group III-proteins: Proteins with depressed relative abundance under co-culture with central glia (SCNN)

Group IV-proteins: Proteins with high relative abundance under local co-culture with central nervous system cells (VSC).



Legend to Fig. 11

Digitized images of 2-dimensional electropherograms indicating proteins with similar modulation profiles.

a) Group I-proteins: Proteins with high relative abundance in the absence of any co-cultured cells (none).

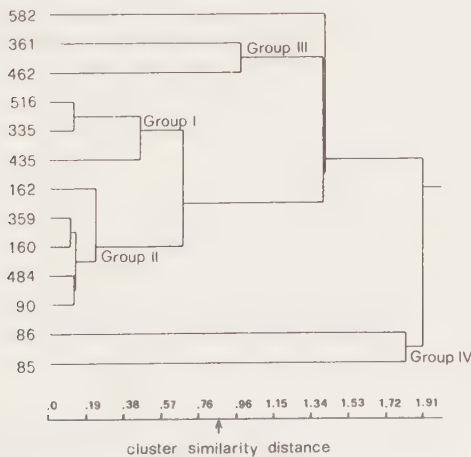
b) Group II-proteins: Proteins with high relative abundance under local co-culture with peripheral glia (DRGNN).

c) Group III-proteins: Proteins with depressed relative abundance under co-culture with central glia (SCNN).

d) Group IV-proteins: Proteins with high relative abundance under local co-culture with central nervous system (VSC).

(Fig. 12). Two of the "co-culture-depressed proteins" (group I), namely 516 and 335, are grouped close together and clearly separated from spot 435 and even further distant from spot 582. Similarly four of the "peripheral glia-enhanced proteins" (group II), namely spots 359, 160, 484 and 90, are grouped very close together and clearly distant from spot 162. The pairs of proteins grouped together as "central glia-depressed proteins" (group III) and "central nervous system cells-enhanced proteins" (group IV) both are revealed as closest to each other, but in both pairs the cluster of the similarity distance is well beyond the cut-off distance.

The modulated proteins that displayed conspicuously similar modulation profiles when subjected to a variety of environmental conditions might be involved in the same axonal functions, undergoing co-regulation under appropriate environmental stimuli. However, parallel behaviour of different functions under the relatively few environmental con-



Legend to Fig. 12

Dendrograms representing the clustering pattern of the 13 environmentally modulated proteins. A clustering algorithm, in general terms, is applied to feature data of N objects each having values for K features (Anderberg, 1973; Hartigan, 1975; Shapiro, 1983; Spencer, 1984). In the case of 2D-gel spot data, a set of spots may be searched for cluster formation considering their features exposed under different experimental conditions. The features considered may be a) the mean values of the concentration in different experimental classes or b) the ratios of the mean densities of the same spot in pairs of experimental classes for all permutations of classes. Given N objects (by default the initial unit-sized clusters), the various clustering techniques basically try to find iteratively the most similar existing clusters. This group of two existing clusters is then defined to be a new cluster. Complete clustering is done by repeating this process N-1 times to finally form one cluster from N initial objects. The particular metric definition of "similar" that we use is the K-dimensional Euclidian distance between any two clusters being compared. Each of the K features is autoscaled to a standard deviate scale by subtracting its mean and dividing by its standard deviation computed over the N samples. This permits a uniform comparison of features. Spencer (1984) estimates a new K-dimensional vector for new clusters using a group-weighted average. After generating the N-1 clusters at various levels it is useful to visualize them. This may be done by use of dendrograms, the Delaunay triangulation, Voronoi diagram, etc. (Shapiro, 1983). Such visualization facilitates interpretation of the clusters in the context of the biological problem. The dendrogram display is used here as it was produced automatically as part of the algorithm employed (Spencer, 1984). Computerized cluster analysis was performed with the 13 modulated spots as the objects and ratios of the mean values of all permutations of pairwise comparisons of all experimental classes as the features. The numbers at the left of the tree indicate the spot numbers. The abscissa represents cluster similarity distance as measured in standard error distance. As a cutoff criterion of "significant clusters", the mean cluster similarity distance plus one standard deviate was used (vertical arrow) (Mojena, 1975).

ditions employed is conceivable as well. Future studies will show which of these proteins cooperate in the same axonal function and are possibly modulated by identical mechanisms.

6. CONCLUDING REMARKS

Cellular structures and functions are accomplished by the concerted action of an overwhelming complexity of molecular components. Only recently have experimental tools for the resolution of the macromolecular composition of tissues and body fluids at the molecular level become available. Progress in analytical technology for the detection and isolation of proteins has made it possible to trace proteins from highly complex biological material. In particular, the technique of 2-dimensional SDS-PAGE (O'Farrel, 1975) allows for the first time, the identification and characterization of proteins from heterogeneous cellular material in large numbers and the resolution of single proteins. Theoretically, between 7,000 (O'Farrel, 1975) and 10,000 (Anderson *et al.*, 1981) individual proteins have been estimated to be resolvable on a two-dimensional gel and efforts towards higher resolution and sensitivity are under way. At the same time, recombinant DNA technologies are furthering data on impressive numbers of genes and gene products. As an example, an estimated 30,000 mRNAs are found in the brain (Sutcliffe & Milner, 1984). Even if not all of these mRNAs may be translated to their corresponding cellular proteins at any point in time (Duncan & Conkey, 1982), it is evident that recent technological progress has disclosed information on large numbers of gene products that were only matter of speculation in the past. However, the fact that a large number of cellular proteins can now be detected reminds us very strongly of our limited understanding of the function of individual proteins. In marked contrast to the relative ease of detection of novel proteins, the functional features of a particular protein are more difficult to ascertain. The assignment of functions to proteins has obviously become the limiting step in the elucidation of the mechanisms nature employs in the imple-

mentation of biological functions and certainly is one of challenges of present and future research in cell biology.

The search for proteins that are prominent in some features has long been practiced as a means to initiate the resolution of the molecular complexity in the study of the molecular basis of cellular functions. As a frequently used experimental approach, proteins with specific subcellular location have been selected as candidates for a role in the implementation of functions that are accomplished exclusively at such particular sites. As examples, proteins associated with synaptic membranes (Barondes, 1974; Banker *et al.*, 1974; Wang & Mahler, 1976; Matus *et al.*, 1980) or proteinaceous components of the synaptic vesicle-specific membrane (Matthew *et al.*, 1981) have been identified and studied. In another very successful approach, proteins prominent by their capability to be phosphorylated were identified, their subcellular location determined, and their role in the regulation of neuronal function studied (for a recent review, cf. Kennedy, 1983).

Our specific objective is to contribute to the elucidation of axonal functions of interest in development or regeneration, such as elongation or synapse formation, at the molecular level. Axons, by their particular morphology, provide a means for reduction of the molecular complexity encountered in the study of their functional features. Since most, if not all, axonal proteins are synthesized in the neuronal soma and translocated into the axon by axonal transport, the proteins located in axons are expected to be selectively those involved in axonal functions. However, even in the axons of a neuronal subpopulation (namely cultured DRG neurons), more than 1000 proteins are discernible (P. Sonderegger, in preparation). Many developmental and

regenerative axonal functions have been found to be unique to particular neuronal subpopulations and many functions are subject to environmental control (cf. Section 4 and 5). The analysis of the pattern of axonal proteins of different neuronal subpopulations or under a variety of environmental conditions, in combination with a quantitative assessment of axonal functions, is proposed here as a way to identify proteins that might have a role in particular axonal functions. This selective approach could help to deal with the complexity of the biological system under investigation by guiding our attention to a relatively small number of intriguing proteins. In vitro experiments performed under a similar rationale, revealed a number of proteins termed "growth-associated proteins" (GAPs). Nerve crush experiments on central and peripheral nerves and in a variety of amniotic and anamniotic animals revealed that a number of axonally transported proteins only occurred in situations where axonal regeneration was successfully achieved (for review, cf. Skene, 1984).

Our own work reviewed here deals with the identification of axonal proteins that are preferentially located in particular subclasses of neurons and with the detection of proteins that are predominantly expressed, when the axons were under particular environmental conditions. Axonal proteins preferentially located in particular subclasses of neurons could be involved in functions or behavioral traits that are specific for these neurons. Similarly, proteins expressed predominantly when axons are exposed to particular environmental conditions may be involved in axonal functions active preferentially under these circumstances.

Of course, further refinement of the process of assigning proteins tentatively to functions and activities subserving particular functions will have to come from correlation of

the expression of a protein under observation with the expression of axonal functions in a variety of neuronal subpopulations or functional states. For this purpose, the establishment of quantitative assays for axonal functions will be as important as a further reduction of the complexity of the experimental system to clearly defined types of interacting cells. With regard to the identification and isolation of cells, important progress has come forth over the past decade. A large number of cell type-specific markers have been developed, encompassing the major classes of neurons, astrocytes, and oligodendrocytes and developmentally distinct subsets of these (for review, cf. Schachner, 1982). Such markers, when used for fluorescence activated cell-sorting (for a review, cf. Loken and Stall, 1982) will make available selected populations of neural cells. Making use of these new experimental possibilities for the isolation and purification of distinct cell types, the compartmental cell culture system used in these studies should allow to analyze the macromolecular composition of the axons of virtually any type of neuron and to identify those molecules specific for particular neuronal subpopulations. Furthermore, co-culture of axons with particular glia cell populations or purified neuronal target cells should help to obtain a detailed description of the cell-cell interactions in the nervous system and, hopefully, contribute to the elucidation of the organizational principles acting in the developing nervous system.

7. SUMMARY

The axonal functions that act in the formation of the neuronal network are phenomenologically described as elongation, sprouting, pathfinding and synapse formation. Many of these processes have been shown to exhibit traits specific for neuronal subpopulations and to occur in close interdependence with the tissue that surrounds the growing axons. Little is known about the molecular building blocks underlying axonal functions. In view of the existence of hundreds of axonal proteins, we have developed an approach to the identification of molecules involved in the implementation of particular axonal functions. As a first step, a generally applicable in vitro approach for the quantitative analysis of axonal proteins was developed and used for the identification of axonal proteins which are preferentially expressed in neuronal subpopulations or whose synthesis is subject to environmentally induced changes. A compartmental cell culture system that offers separate access to cell somas and axons was used when dissociated neural cells are plated in the center compartment, their outgrowing axons cross a barrier between center and side compartments through a thin film of medium, whereas the cell somas are retained in the center compartment. The proteins synthesized by the neuronal cell can be metabolically labeled with ^{35}S methionine added to the medium of the center compartment. By harvesting the cellular material of the side compartment, one ensures that the proteins synthesized by the neuronal somas in the center compartment and translocated by axonal transport into axons extending into the side compartments are the only radioactively labeled proteins found in the side compartments. Hence, this system allows selective investigation of proteins of axons even when they are located in a variable cellular environment. Two-dimensional sodium dodecylsulfate-polyacrylamide gel

electrophoresis and computerized quantitation of the individual axonal proteins revealed that the axonal protein patterns of two neuronal subpopulations, namely dorsal root ganglion neurons and ventral spinal cord neurons, are distinct in a few proteins and that co-cultured cells from the peripheral nervous system and the central nervous system modulate the synthesis of a few axonal proteins of dorsal root ganglion neurons differentially. Axonal proteins with preferential occurrence in particular neuronal subpopulations are candidates for proteins with a role in subpopulation-specific functions. Plasticity in the expression of axonal proteins in response to the different topographical provenance of the surrounding cells may reflect environmental modulation of axonal functions during the development of the nervous system.

The capacity to detect axonal proteins specific for neuronal subpopulations and the ability to produce changes in axonal functional properties demonstrating concomitantly altered expression of proteins may be useful in approaching the difficult task to relate axonal proteins to particular axonal functions. Proteins tentatively assigned to certain axonal functions may then be investigated in greater detail by cell biological, biochemical, and recombinant DNA technologies to obtain insight into the molecular details of the processes acting in the implementation and regulation of axonal functions.

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REFERENCES

- Anderson, N.L., Taylor, J., Scandora, A.E., Coulter, B.P. and Anderson, N.G., The TYCHO system for computer analysis of two-dimensional gel electrophoresis patterns. Clin. Chem., 27, (1981) 1807-1820.
- Altschuler, R.A., Mosinger, J.I. Harmison, G.G., Parakkal, M.H. and Wenthold, R.J., Aspartate aminotransferase-like immunoreactivity as a marker for aspartate/glutamate in guinea pig photoreceptors. Nature (Lond), 298 (1982) 657-659.
- Anderberg, M.R., Cluster analysis for applications, Academic Press, New York (1973) Chapter 6.
- Banker, G., Churchill, L. and Cotman, C.W., Proteins of the postsynaptic density, J. Cell Biol. 63 (1974) 456-465.
- Baron van Evercooren, A., Kleinman, H.K., Ohno, X., Marangos, P., Schwartz, J.P. and Dubois, Dalq, M.E., Nerve growth factor, laminin and fibronectin promote neurite growth in human fetal sensory ganglia cultures. J. Neurosci. Res. 8, (1982) 179-183.
- Barondes, S.H., Synaptic macromolecules: Identification and metabolism. Ann. Rev. Biochem. 43 (1974) 147-168.
- Black, M.M. and Lasek, R.J., The presence of transfer RNA in the axoplasm of the squid giant axon, J. Neurobiol., 8 (1977) 229-237.
- Bondy, S.C. and Purdy, J.L., Migration of ribosomes along the axons of the chick visual pathway, Biochem. Biophys. Acta 390, (1975) 332-341.

- Bonner, W.M. and Laskey, R.A., A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem., 46 (1974) 83-88.
- Braun, S.J., Sweadner, K.J. and Patterson, P.H., Neuronal cell surfaces: Distinctive glycoproteins of cultured adrenergic and cholinergic sympathetic neurons. J. Neurosci., 1 (1981) 1397-1406.
- Bray, D., Wood, P. and Bunge, R.P., Selective fasciculation of nerve fibers in culture, Exp. Cell Res., 130 (1980) 241-250.
- Campenot, R.B., Local control of neurite development by nerve growth factor. Proc. Natl. Acad. Sci. USA, 74 (1977) 4516-4519.
- Campenot, R.B., Independent control of the local environment of somas and neurites, Methods Enzymol., 58 (1979) 302-307.
- Celio, M.R. and Heizmann, C.W., Calcium-binding protein parvalbumin as a neuronal marker, Nature, 293 (1981) 300-302.
- Chalazonitis, A. and Fischbach, G.D., Elevated potassium induces morphological differentiation of dorsal root ganglionic neurons in dissociated cell culture. Dev. Biol., 78 (1980) 173-183.
- Cohen, J. and Selvendran, S.Y., A neuronal cell-surface antigen is found in the CNS but not in peripheral neurons, Nature (Lond), 291 (1981) 421-423.

David, S. and Aguayo, A.J., Axonal elongation into peripheral nervous system injury in adult rats, Science 214 (1981) 931-933.

Duncan, R. and McConkey, E.H., How many proteins are there in a typical mammalian cell?, Clin. Chem., 28 (1982) 749-7655.

Edgar, D., Timpl, R. and Thoenen, H., The heparin-binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival, EMBO J. 3 (1984) 1463-1468.

Edstrom, A. and Sjostrand, J., Protein synthesis in the isolated Mauthner nerve fiber of goldfish, J. Neurochem., 16 (1969) 67-81.

Greene, L.A., The importance of both early and delayed responses in the biological actions of nerve growth factor, Trends Neurosci., 7, 91-94.

Fields, K.L., Brookes, J.P., Mirsky, R. and Wendon, L.M.B., Cell surface markers for distinguishing different types of rat dorsal root ganglion cells in culture, Cell, 14 (1978) 43-51.

Fischbach, G.D., Synapse formation between dissociated nerve and muscle cells in low density cell cultures., Dev. Biol., 28 (1972) 407-429.

Fischbach, G.D. and Nelson, P.G., Cell cultures in neurophysiology. In: Handbook of Physiology. The Nervous System I, Chapter 20 (1977) pp. 719-779. Am. Physiol. Soc., Washington D.C.

Frankel, R.D. and Koenig, E., Identification of locally synthesized proteins in proximal stump axons of the neurotomized hypoglossal nerve. Brain Res. Rev., 141 (1978) 67-76.

Fuchs, P.A., Nicholls, J.G. and Ready, D.F., Membrane properties and selective connections of identified leech neurons in culture, J. Physiol., 316 (1981) 204-223.

Gottlieb, D.I. and Glaser, L., Cellular recognition during neural development, Ann. Rev. Neurosci., 3 (1980) 303-318.

Gundersen, R.W. and Barrett, J.N., Neuronal chemotaxis: Chick dorsal root axons turn toward high concentrations of nerve factor, Science, 206 (1979) 1079-1080.

Hamburger, V. and Hamilton, H.L., A series of normal stages in the development of the chick embryo, J. Morphol., 88 (1951) 49-92.

Hartigan, J.A., Clustering algorithms, John Wiley & Sons, New York (1975).

Hattan, M.E. and Sidman, R.L., Plant lectins detect age and region differences in cell surface carbohydrates and cell reassociation behaviour of embryonic cerebellar cells, J. Supramol. Struct., 7 (1977) 267-275.

Hattan, M.F., Schachner, M. and Sidman, R.L., Histochemical characterization of lectin binding in mouse cerebellum., Neuroscience, 4 (1979) 921-935.

Holliday, M. and Hamburger, V., Autoradiographic study of the formation of the lateral motor column in the chick embryo., Brain Res., 132 (1977) 197-209.

Kao, C.C., Chang, L.W. and Bloodworth, Jr., J.M.B., Axonal regeneration across transected mammalian spinal cords: An electron microscopic study of delayed microsurgical nerve grafting, Exp. Neuro., 54 (1977) 591-615.

Kennedy, M.B., Experimental approaches to understanding the role of protein phosphorylation in the regulation of neuronal functions, Ann. Rev. Neurosci. 6 (1983) 493-525.

Koenig, E., Ribosomal RNA in Mauthner axon: Implication for a protein synthesizing machinery in the myelinated axon, Brain Res., 174 (1979) 95-107.

Krystosek, A. and Seeds, N.W., Plasminogen activator secretion by granula neurons in cultures of developing cerebellum, Proc. Acad. Sci. 78 (1981a) 7810-7814.

Krystosek, A. and Seeds, N.W., Plasminogen activator release at the neuronal growth cone, Science 213 (1981b) 1532-1534.

Landmesser, L.T., The generation of neuromuscular specificity, Ann. Rev. Neurosci. 3 (1980) 279-302.

Landmesser, L., The development of specific motor pathways in the chick embryo, Trends Neurosci. 7 (1984) 336-339.

Lemkin, P.F., Lipkin, L.E. and Lester, E.P., Some extensions to the GELLAB two-dimensional electrophoretic gel analysis system, Clin. Chem., 28 (1982) 840-849.

Lemkin, P.F., Sonderegger, P. and Lipkin, L.E., Identification of coordinate pairs of polypeptides: A technique for screening of putative precursor product pair in 2D gels, Clin. Chem. 30 (1984) 1965-1971.

Lemkin, P.F. and Lipkin, L.E., 2D-Electrophoresis gel data base analysis: Aspects of data structures and search strategies in GELLAB, Electrophoresis, 4 (1983a) 71-81.

Lemkin, P.F. and Lipkin, L.E., Database techniques for two-dimensional electrophoretic gel analysis. In M. Geisow and A. Barret (Eds.), Computing in Biological Science, Elsevier North Holland, Amsterdam, 1983b, pp. 181-231.

Letourneau, P.C., Cell-to-stratum adhesion and guidance of axonal elongation, Dev. Biol., 44 (1975) 92-101.

Letourneau, P.C., Nerve fiber growth and its regulation by extrinsic factors. In N.C Spitzer (Ed.), Neuronal Development, Plenum Press, New York, 1982.

Levi-Montalcini, R., Meyer, H. and Hamburger, V., In vitro experiments on the effects of mouse sarcomas 180 and 37 on the spinal and sympathetic ganglia of the chick embryo, Cancer Res., 14 (1954) 49-57.

Lipkin, L.E. and Lemkin, P.F., Data-base techniques for multiple two-dimensional polyacrylamide gel electrophoresis analysis, Clin.Chem., 26 (1980) 1403-1412.

Loken, M.R. and Stall, A.M., Flow cytometry as an analytical and preparative tool in immunology. I. Immunol. Meth., 50 (1982) R 85-R 112.

Matthew, W.D., Tsavaler, L. and Reichardt, L.F., Identification of a synaptic vesicle-specific membrane protein with wide distribution in neuronal and neurosecretory tissue, J. Cell Biol. 91 (1981) 257-269.

Matus, A., Pehling, G., Ackermann, M. and Maeder, J., Brain postsynaptic densities: Their relationship to glial and neuronal filaments, J. Cell Biol. 87 (1980) 346-359.

McMillan Carr, V. and Simpson, Jr., S.B., Proliferative and degenerative events in the early development of chick dorsal root ganglia, J. Comp. Neurol. 182 (1978) 727-740.

Mojena, R., Hierarchical grouping methods and stopping rules: An evaluation, The computer Journal 20 (1975) 359-363.

Mudge, A.W., Effect of chemical environment on levels of substance P and somatostatin in cultured sensory neurons, Nature, 292 (1981) 764-767.

Mudge, A.W., Schwann cells induce morphological transformation of sensory neurons in vitro, Nature, 309 (1984) 367-369.

O'Farrel, P.H., High resolution two-dimensional electrophoresis, J. Biol. Chem. 250 (1975) 4007-4021.

Omlin, F.X., Matthieu, J.-M., Philippe, E., Roch, J.-M., and Droz, B., Expression of myelin-associated glycoprotein by small neurons of the dorsal root ganglion in chickens, Science 227 (1984) 1359-1360.

Patterson, P.H., Environmental determination of autonomic neurotransmitter function, Ann. Rev. Neurosci., 1 (1978) 1-17.

Perkins, C.S., Carlstedt, T., Mizuno, K. and Aguayo, A.J., Failure of regenerating dorsal root axons to regrow into the spinal cord, Can. J. Neurol. Sci. 8 (1980) 323.

Pfenninger, K.H. and Maylie-Pfenninger, M.F., Lectin labeling of sprouting neurons. I., Regional distribution of surface glycoconjugates, J. Cell Biol. 89 (1981) 536a.

Ramon y Cajal, S., Degeneration and regeneration of the nervous system (translated by R.M. May), Oxford Press, London (1928).

Ransom, B.R., Christian, C.N., Bullock, P.N. and Nelson, P.G., Mouse spinal cord in cell culture. II. Synaptic activity and circuit behaviour, J. Neurophysiol. 40 (1977) 1151-1162.

Raper, J.A., Bastiani, M. and Goodman, C.S., Pathfinding by neuronal growth cones in grasshopper embryos. II. Selective fasciculation onto specific axonal pathways, J. Neurosci. 3 (1983a) 31-41.

Raper, J.A., Bastiani, M. and Goodman, C.S., Pathfinding in neuronal growth cones in grasshopper embryos. I Divergent choices made by the growth cone of sibling neurons, J. Neurosci. 3 (1983b) 20-30.

Reier, P. J., Stensaas, L.J. and Guth, L., The astrocytic scar as an impediment to regeneration in the central nervous system. In C.C. Kao, R.P. Bunge and P.J. Reier (Eds.), Spinal Cord Reconstruction, Raven Press, New York, 1983.

Richardson, P.M., McGuinness, U.M. and Aguayo, A.J., Axons from CNS neurons regenerate into PNS grafts, Nature 284 (1980) 264-265.

Schachner, M., All type-specific surface antigens in the mammalian nervous system, J. Neurochem. 39 (1982) 1-8.

Rutishauser, U., Gall, W.E. and Edelman, G.M., Adhesion among neural cells of the chick embryo. IV. Role of the cell surface molecule CAM in the formation of neurite bundles in cultures of spinal ganglia, J. Cell Biol., 79 (1978) 382-393.

Schwab, M. and Landis, S., Membrane properties of cultured rat sympathetic neurons: Morphological studies of adrenergic and cholinergic differentiation, Dev. Biol., 84 (1981) 67-78.

Schwab, M., and Thoenen, H., Mechanism of uptake and retrograde axonal transport of noradrenaline in sympathetic neurons in culture: reserpine resistant large dense-core vesicles as transport vehicles, J. Cell Biol., 96 (1983) 1538-1547.

Snapiro, M., C-LAB applications manual; 1st edition. LSM, DCRT, NIH, Bethesda, MD 20205, 1983.

Skene, P.J.H., Growth-associated proteins and the curious dichotomies of nerve regeneration. Cell 37 (1984) 697-700.

Sonderegger, P., Fishman, M.C., Bauer, H.C. and Nelson, P.G., Axonal proteins of presynaptic neurons during synaptogenesis, Science, 221 (1983) 1294-1297.

Sonderegger, P., Fishman, M.C., Bokoum, M., Bauer, H.C., Neale, E.A. and Nelson, P.G., A few axonal proteins distinguish ventral spinal cord neurons from dorsal root ganglion neurons, J. Cell Biol., 98 (1984) 364-368.

Sonderegger, P., Lemkin, P.F., Lipkin, L.E. and Nelson, P.G., Differential modulation of the expression of axonal

proteins by non-neuronal cells of the peripheral and central nervous system, EMBO J., in press.

Spencer, B., Cluster analysis, Byte, (1984) 129-130 and 423-426

Sperry, R.W., Chemoaffinity in the orderly growth of nerve fiber patterns and connections. Proc. Natl. Acad. Sci. USA, 50 (1963) 703-710.

Stelzner, D.J., Martin, A.H. and Scott, G.L., Early stages of synaptogenesis in the cervical spinal cord of the chick embryo, Z. Zellforsch. Mikrosk. Anat., 138 (1973) 475-488.

Stensaas, L.J., Burgess, P.R. and Horch, K.W., Regenerating dorsal root axons are blocked by spinal cord astrocytes, Soc. Neurosci. Abstr., 5 (1979) 684.

Sugar, O. and Gerard, R.W., Spinal cord regeneration in the rat, J. Neurophysiol. 3, (1940) 1-19.

Suttcliffe, J.G. and Milner R.J., Brain specific gene expression, Trend Neurosci., 7 (1984) 95-99.

Sweadner, K.J., Environmentally regulated expression of soluble extracellular proteins of sympathetic neurons. J. Biol. Chem., 256 (1981) 4063-4070.

Vulliamy, T., Rattray, S. and Mirsky, R., Cell-surface antigen distinguishes sensory and autonomic peripheral neurones from central neurones, Nature 291 (1981) 418-420.

Wang, Y.-J. and Mahler, H.R., Topography of the synaptosomal membrane, J. Cell Biol. 71 (1976) 639-658.

Wilson, D.L. and Stone, G.C., Axoplasmic transport of proteins, Ann. Rev. Biophys. Bioeng., 8 (1979) 27-45.

Wilson, S.H., Schrier, B.K., Farber, J.L., Thomson, E.J., Rosenberg, R.N., Blume, A.J. and Nirenberg, M.W., Markers for gene expression in cultured cells from the nervous system, J. Biol. Chem., 247 (1972) 3159-3169.

Wood, P.M., Separation of functional Schwann cells and neurons from normal peripheral nerve tissue, Brain Res., 115 (1976) 361-375.

Zurn, A.D., Identification of glycolipid binding sites for soybean agglutinin and differences in the surface glycolipids of cultured adrenergic and cholinergic sympathetic neurons, Dev. Biol., 94 (1982) 483-498).

